

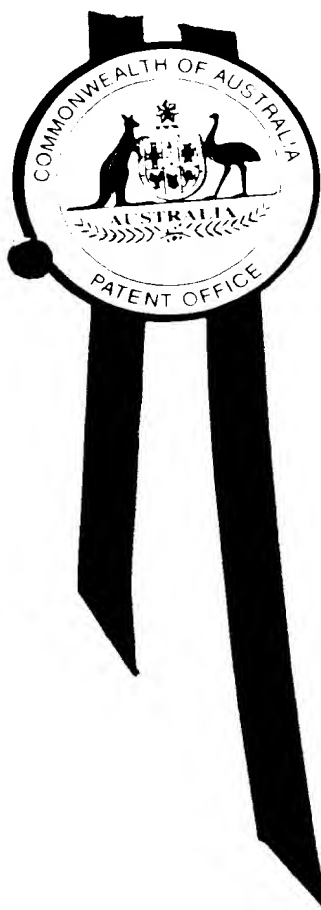


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PROVISIONAL SPECIFICATION

Invention Title:

Regulatory element II

The invention is described in the following statement:



Regulatory element II

FIELD OF THE INVENTION

The present invention relates to constructs comprising a novel regulatory element derived from a prostate specific gene. The present invention also relates to diagnostic and therapeutic methods involving the use of these constructs.

BACKGROUND OF THE INVENTION

The isolation and characterisation of DNA regions which control tissue specific and/or hormonally-regulated gene expression has been an important to the understanding of the developmental processes by which expression of particular genes is limited to specific cell types. Promoter regions are found immediately upstream and often overlapping the start site(s) of transcription and are critical for initiation and basal levels of transcription. Enhancers are regulatory regions which may lie some distance from the transcription start site, either upstream or downstream of a gene or within introns and which often confer high level tissue specific or hormonally-regulated expression; in some cases their action is specific to particular promoters. The function of both promoters and enhancers is mediated by specific proteins, transcription factors, that bind to specific DNA sequences. Alone or in combination with other transcription factors they recruit the core transcription machinery including RNA polymerase to the transcription initiation site and act to stimulate their activity. Isolated promoters and enhancer sequences can be used, in gene therapy for example, to direct expression of other genes in a cell or tissue specific manner and also provide targets for the development of agents that can specifically modulate gene expression.

The promoters and regulatory regions of a number of genes that are expressed in the prostate have been studied either using transfection techniques or by following gene expression in transgenic mice. We have previously compared the cell-type specificity of expression directed by promoters of the prostate-expressed genes, probasin (Pb) and relaxin genes and the promoter and enhancer of the prostate specific antigen (PSA) gene (1). Most of the genes identified as prostate-specific are androgen-inducible and this aspect of their function has been studied in some detail. Thus the importance

of androgen response elements for induced expression and/or binding of androgen receptor have been characterised in the PSA (2, 3), human glandular kallikrein (KLK2) (4), rat prostatic steroid binding proteins (PSBP) (5, 6), probasin Pb (7, 8) and prostatic acid phosphatase genes (9) and in regulatory elements in the introns of the rat PSBP C3(1) gene (10) and the rat 20-KDa androgen regulated protein (11).

Among the core promoter regions analysed only that of the probasin gene confers substantial prostate specificity of expression (1, 15). Elements involved in conferring prostate-specificity of expression per se, as distinct from androgen responsiveness, have not been well characterised, though tissue-specific factors binding to regions of the PSBP C3 gene promoter and 1st intron have been identified (9, 12). The gene for rat PSBP C(3) with 4 kb upstream and 2 kb downstream flanking sequences is expressed tissue-specifically and with appropriate hormonal control in transgenic mice (13). The use of a 5kb upstream region from the rat PSBP C3(1) gene to express the SV40 T-antigen could elicit prostate tumours, but expression was not highly restricted and other abnormalities were common (14). Studies with transgenic mice have established that regions of the probasin and PSBP C(3) genes can confer prostate specificity.

The PSA and probasin regulatory regions are the two most studied among prostate-expressed genes. It has been established that a 430 bp region upstream of the rat probasin gene is able to confer prostate specificity of expression on reporter genes in transfection experiments (1) and in transgenic animals (15, 16); when used to target expression of the SV40 T-antigen, prostate tumours develop specifically (17, 18). This expression is not totally specific but specificity is significantly improved by the inclusion of MAR (matrix attachment regions) from the chick lysozyme gene (15). The 430 bp promoter region is strongly responsive to androgen induction and androgen response elements which bind the androgen receptor (AR) have been characterised (4, 6, 7, 16).

The PSA upstream region (to -630 bp) also acts as a strongly androgen responsive promoter and androgen response elements have also been characterised (2, 3). However, this region is not sufficient to direct cell type specific expression in culture (1) or tissue specific expression in transgenic mice (19). Use of the 630 bp human PSA promoter region to express an activated Ha-ras oncogene in transgenic mice led to the development of salivary

gland and not prostate tumours (19). Pang et al. have reported that the equivalent promoter region isolated from a prostate cancer patient contained 7 mutations compared to the published sequence and was highly active in the prostate cancer cell line LNCaP (20, 21). More recently, an enhancer region has been identified in the region 4 to 5kb upstream of the transcription start site of the PSA gene (20, 21). This PSA enhancer has been shown to act as an androgen-inducible enhancer and in combination with the PSA promoter to display significant cell-type specificity (1, 20, 21).

Prostate-Specific Membrane Antigen

Prostate specific membrane antigen (PSMA) is one of the few prostate-specific proteins identified whose expression is not induced by androgens. PSMA was first identified as the antigen bound to by the monoclonal antibody 7E11-C5(25). The antibody was raised against a membrane fraction of the prostate cancer cell line LNCaP and was shown to bind specifically to normal prostate tissue as well as primary and metastatic prostate cancer tissue. This antibody was later found to bind to an internal epitope of this membrane-bound protein (26, 27). Subsequently, other monoclonal antibodies targeted to the extracellular domain of the protein have been isolated (28, 29).

The cDNA encoding PSMA has been cloned and its sequence determined (30). PSMA is a Type II integral membrane protein and is associated with the plasma membrane of expressing cells such as LNCaP (30). A splice variant of PSMA (Psm') that lacks the membrane anchor domain and has been shown to be cytoplasmically located has also been identified (31). The ratio of PSMA to Psm' has been reported to be increased in prostate cancer as compared with normal prostate or benign hyperplasia (31). PSMA has been shown to possess two related enzymatic activities, it acts as a carboxypeptidase (folate hydrolase) on poly γ -glutamated folates (32) and as a peptidase on the acidic neuropeptide N-acetylaspartyl glutamate (33). This latter activity is consistent with the expression of PSMA or a related protein in the brain.

The specificity of PSMA expression has been studied at both the protein and RNA level. In addition to its major site of expression in the prostate immunohistochemical studies have identified PSMA expression in the duodenum brush border/small intestine, in a subset of proximal tubules in the kidney and in rare cells in the colon (34, 35). All other normal tissues studies have been negative for expression, except for striated muscle which stains with

the 7E11-C5 antibody, but not with antibodies to the external domain of PSMA (28).

Both the 7E11-C5 and external domain antibodies have been found to react with tumour vasculature of a wide range of human tumour types (28, 36), indicating specific induction of PSMA expression. PSMA expression has not been identified in any normal vasculature.

RNA expression has been found to largely parallel the protein expression data. RNase protection analysis identified PSMA mRNA in the prostate, salivary gland and brain and sometimes in the small intestine (37). The identification of PSMA RNA in the brain is consistent with the cloning of a closely related cDNA from rat brain (33). Immunohistochemical analyses have failed, however, to identify antigenically reactive PSMA in human brain tissue.

PSMA expression has been shown to be down regulated in the presence of androgens and expression is generally elevated in late stage prostate cancer and in patients undergoing androgen deprivation or ablation therapies (37, 38). Expression of PSMA has also been found to be regulated by a number of growth factors: bFGF, TGF- α and EGF upregulate expression while TNF- α decreases it (39).

The restricted high level expression of PSMA in prostate cells and the induction of its expression in the vasculature of a wide range of tumours make it ideal for the targeting of prostate and other tumour types. Genomic clones encompassing the PSMA gene have been isolated and its sequence and exon/intron structure determined (40). Regulatory regions controlling its expression may find use in gene therapeutic cancer treatments, enabling the restricted or high level expression in the target cell types. Such regulatory regions also provide a target for the development of agents that may interfere with gene expression in the target cell types.

SUMMARY OF THE INVENTION

The present inventors have identified a novel regulatory element in the PSMA gene. The regulatory element is an enhancer located in intron 3 of the PSMA gene. This is the first report, to the applicant's knowledge, of an enhancer derived from an androgen-independent, prostate specific gene.

When used herein, the PSMA gene refers to the PSMA genomic sequence described in *O'Keefe et al, 1998* (40), the entire contents of which are incorporated herein by reference.

Accordingly, in a first aspect the present invention provides a recombinant DNA molecule comprising a sequence encoding an RNA transcript or polypeptide other than the prostate specific membrane antigen (PSMA) transcript or polypeptide and at least one regulatory element derived from intron 3 of the PSMA gene.

In a preferred embodiment, the recombinant DNA molecule further comprises a promoter. Preferably, the promoter is located upstream from and is operably linked to the sequence encoding the polypeptide.

In a second aspect, the present invention provides a recombinant expression cassette comprising a regulatory element derived from intron 3 of the PSMA gene, a promoter, and an insertion site into which a coding sequence can be inserted, the insertion site being adjacent to and downstream of the promoter.

The regulatory element(s) may be located in either orientation anywhere within the recombinant DNA molecule or expression cassette of the present invention. For example, the regulatory element may be located downstream of the coding sequence (eg. downstream of the 3' termination or polyadenylation signals) or within an intron located in the coding sequence. In a preferred embodiment, the regulatory element is located adjacent to the promoter. More preferably, the regulatory element is upstream of the promoter.

Within the context of the present invention, it is preferred that the regulatory region is an enhancer element. Preferably, the enhancer element includes intron 3 of the PSMA gene. More preferably, the enhancer element is derived from intron 3 between nucleotides 14,045 and 16,575 of the PSMA gene.

In a preferred embodiment, the enhancer element comprises the sequence from nucleotides 14760 to 14930, more preferably from nucleotides 14760 to 15091.

In another embodiment, the enhancer element comprises the sequence from (i) nucleotides 14,045 to 15,804, (ii) nucleotides 14,760 to 15,804 or (iii) nucleotides 14,760 to 16,575 of the PSMA gene. The enhancer element may comprise the sequence from nucleotides 14,045 to 16,575 of the PSMA gene.

In a further preferred embodiment the recombinant DNA molecule or expression cassette of the present invention comprises two or more regulatory elements derived from intron 3 of the PSMA gene. In one preferred embodiment, the recombinant DNA molecule or expression cassette comprises a dimer of a regulatory element derived from intron 3 of the PSMA gene.

It will be appreciated by those skilled in the art that any suitable promoter may be used in the context of the present invention. Preferred promoters include, but are not limited to, herpes virus thymidine kinase (TK), and Rous sarcoma virus (RSV) promoters, promoters active in the prostate, such as probasin, PSMA, and PSA, or promoters active in vascular endothelium.

In a preferred embodiment, the the recombinant DNA molecule and expression cassette of the present invention further comprise a polyadenylation signal located downstream from and operably linked to the the sequence encoding the polypeptide, or downstream from the insertion site. Preferably, the polyadenylation signal is the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal as described in US 5122458, the entire contents of which are incorporated herein by reference.

In a third aspect, the present invention provides a vector comprising a recombinant DNA molecule of the first aspect or an expression cassette of the second aspect.

In one preferred embodiment the vector comprises a gene encoding a selectable marker. The vector may further include an origin of replication.

It is presently preferred that the vector is human adenovirus Type 5 or ovine adenovirus.

In a fourth aspect the present invention provides a method for directing expression of a coding sequence of interest in a cell, the method comprising introducing into the cell a recombinant expression cassette comprising at least one regulatory element derived from intron 3 of the PSMA gene, a promoter, and a coding sequence, wherein the regulatory element and promoter direct expression of the coding sequence.

In a preferred embodiment of the fourth aspect, the cell is a prostate cell, bladder cell, breast cell or vascular endothelial cell.

In a fifth aspect the present invention provides a method of treatment of cancer which method involves administering to a subject a recombinant expression construct of the second aspect of the present invention.

As the constructs of the present invention are useful for expression of proteins in vascular endothelial cells, a range of cancer types may be treated within the context of the fifth aspect of the present invention. In a preferred embodiment of the fifth aspect, however, the cancer is selected from prostate, bladder or breast cancer.

As will be appreciated by those skilled in the field, the present invention provides novel regulatory elements from a gene expressed specifically in prostate, which are active both in the presence and absence of androgens. These regulatory elements may therefore be used for high level gene expression in prostate cells. Combinations of one or more of the regulatory elements with the probasin and PSA promoters are examples of constructs that provide for high level expression with strong prostate specificity.

The regulatory elements of the present invention may also be useful for directing expression in a limited range of other cell types, including tumour neovasculature and kidney cells.

The regulatory elements of the present invention may be used to target specific expression of genes to prostate cells or tumour neovasculature or kidney cells in gene therapy.

The regulatory elements of the present invention may also be used to target specific expression of genes in the development of transgenic animal models of prostate disease.

The regulatory elements of the present invention may also be used in assays to identify reagents that interfere with prostate gene expression, or to identify proteins and other factors involved in regulation of prostate gene expression.

Throughout this specification, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. pPSMentrap Vector. Key features of the vector are shown: the multicloning site (MCS) unique restriction sites upstream of the PSM 1k promoter region (PSM1k), leader sequence and intron (intron) derived from the pCI vector(Promega), the green fluorescent protein gene (GFP) and 3' sequences derived from the bovine growth hormone gene (bGHpA). A selection of useful restriction enzyme sites are shown; unique restriction enzyme sites are shown in bold.

Figure 2. Location of cloned PSM enhancer fragments: the map shows the location of the cloned enhancer fragments within intron 3 of the PSM gene. Base numbers (Genbank Accession No. AF007544) are indicated for the boundaries of intron 3 and for the ends of the cloned segments. The locations of the restriction sites SmaI (Sm), HindIII (H) and SpeI (Sp) within the intron are shown. The arrows indicate the orientation of the cloned sequences within the pPSMentrap vector (see Figure 3). The right hand end of the enhancer clone #1 is shown as a stippled box since this end of the clone has undergone rearrangement; The SmaI, HindIII and SpeI sites are present in all three cloned regions.

Figure 3. Promoter and enhancer inserts in pPSMentrap: The positions of the PSM 1kb promoter region and flanking restrictions sites in pPSMentrap are shown on the top line. To the right of the promoter sequences are the leader sequence and chimeric intron and GFP reporter gene. Below are shown maps of clones containing the En3 and En4 inserts. The sequences are in opposite orientation (note order of HindIII and SpeI sites). Restriction sites are abbreviated as follows:

B2 BglII	E EcoRI	H HindIII	K KpnI	M MfeI	N NsiI
Nh NheI	P PstI	S SalI	Sp SpeI	X XbaI	

Figure 4. Promoter and enhancer inserts in pCAT3SAT. Maps show the positions of the PSM 1 kb promoter, PSM En4 and the RSV promoter and their flanking restriction enzyme sites. To the right of the promoters is the leader

sequence and chimeric intron and CAT reporter gene as present in the Promega pCAT3 Basic vector. Restriction enzyme sites are abbreviated as follows:

B2 BglII	Bz BstZI	E EcoRI	H HindIII	K KpnI	M MfeI
MI MluI	N NsiI	Nh NheI	P PstI	S SalI	Sc SacI
Sm SmaI	Sp SpeI	X XbaI	Xh XhoI		

Figure 5. Relative CAT expression directed by the PSM Enhancer4/PSM1k promoter. Following transfection of pPSM1k-C3S or pEn4PSM1k-C3S into the cell lines indicated normalised expression levels were determined for each construct and are expressed relative to that determined from transfection of the pRSV-C3S plasmid.

Figure 6. Promoter and enhancer inserts in pGL3. Maps show the position and flanking restriction enzyme sites of the PSM 1kb promoter (shaded boxes). PSM enhancer fragments (solid boxes) and the RSV promoter (diagonal shading) in the different constructs prepared in the pGL3 vector. To the right of the region shown is the leader and chimeric intron and luciferase reporter gene of the pGL3 vector. PEN4PSM1k-GL3 and pEn3PSm1k-GL3 contain sequences of enhancer clones #4 and #3 respectively as shown in Figure 2.

pEn3+4PSM1k-GL3 contains PSM enhancer sequences encompassing bases 14,045 to 16,575 (see Figure 2). POverlap3.4aPSM1k-GL3 and pOverlap3.4bPSM1k-GL3 contain enhancer sequences from bases 14,760 to 15,804, the a and b constructs containing the enhancer sequences in opposite orientations as indicated by the position of the HindIII and SpeI sites.

Restriction enzyme sites are abbreviated as follows:

A ApoI	B2 BglII	Bz BstZI	E EcoRI	Eo EcoO109I	H HindIII
K KpnI	M MfeI	MI MluI	N NsiI	Nh NheI	Nt NotI
P PstI	RV EcoRV	S SalI	Sc SacI	Sc2 SacII	Sm SmaI
Sp SpeI	X XbaI	Xh XhoI			

Figure 7. Relative luciferase expression of PSM enhancer/promoter constructs in the pGL3 vector. Mixtures of luciferase reporter plasmids (1.5 µg) and the normalising plasmid pRSV-CAT (1 µg) were transfected into different cell lines as shown. Normalised luciferase expression was determined and activity of the different plasmids expressed relative to the normalised expression from pRSV-GL3. Numbers above the columns indicate the relative

enhancement of activity compared with expression from the PSM promoter alone construct, pPSM1k-GL3.

Figure 8. PSM enhancer constructs with other promoters. Maps show the positions and flanking restriction enzyme sites of the PSM enhancer sequences (En4, solid boxes), and promoters from the PSA (diagonal pattern), probasin (vertical pattern) and thymidine kinase (horizontal pattern) genes. To the right of the promoters is the CAT reporter gene of the pCATSAT vector. Restriction enzyme sites are abbreviated as follows:

B BamHI	B2 BglII	E EcoRI	H HindIII	N NsiI	P PstI
S Sall	Sm SmaI	Sp SpeI	X XbaI		

Figure 9. Relative enhancement of heterologous promoters by PSM En4.

- a. Prostate cell lines
- b. Non-prostate cell lines

The different promoter and enhancer constructs were transfected into cell lines as shown and CAT reporter gene expression normalised against SAT expression determined. Activities are expressed as a percentage of the normalised expression of pRSV-CAT. Numbers above the columns indicate the relative enhancement of activity compared with expression from the respective promoter alone constructs. An * indicates that expression levels were too low to determine a ratio.

Figure 10. Effect of androgen on enhancement of heterologous promoters by PSM En4. Plasmids containing the different enhancer/promoter combinations as indicated below the graph were transfected into LNCaP cells that were maintained in medium that had been charcoal stripped to remove androgens or in equivalent medium to which the non-metabolizable androgen analogue R1881 had been added to 0.28 nM. The presence or absence of androgen is also indicated (- or +) below the graph. Activities were determined and expressed as described in Figure 9.

Figure 11. Sequence of 331 base pair core region of the PSME

Figure 12. GFP expression directed by the PSM enhancer/promoter.

DETAILED DESCRIPTION OF THE INVENTION

Isolation of PSMA gene enhancer sequences

Analyses of the region upstream and encompassing the transcription start site of the PSMA gene (40) has shown that a 1kb region directs expression of reporter genes in the prostate cell line LNCaP. This expression shows specificity for prostate cells when compared to that directed by the SV40 enhancer/promoter. Expression in LNCaP cells was about 75% of that directed by the SV40 enhancer/promoter. Comparison with another widely expressed promoter, that of the Rous sarcoma virus (RSV) has indicated that the SV40 enhancer/promoter is only very weakly active, <1% of RSV activity, in LNCaP cells (unpublished data). We have cloned regions encompassing up to 11kb of sequences 5' to the PSMA transcription start site and tested their ability to provide increased reporter gene expression; no increased activity was seen relative to the 1 kb promoter region.

A strategy was developed to allow screening of DNA fragments for their ability to enhance transcription directed by the 1 kb proximal promoter region of the PSMA gene. The 1 kb promoter was cloned in front of the Green Fluorescent Protein (GFP) gene in the plasmid vector pPSMentrap shown in Figure 1. Upstream of the promoter was inserted a polylinker region containing sites for cloning candidate fragments.

pPSMentrap contains the following elements: a polylinker containing restriction sites for the enzymes Kpn1, HindIII, SalI, MfeI, NsiI, BglI, NheI and SpeI, the PSMA promoter region stretching from base 1386 base 2560 (XbaI site) of the PSMA sequence (Genbank Accession No. AF007544), a chimeric intron as contained in the pC1 vector (Promega), the GFP gene, the 3' end polyadenylation signal from the bovine growth hormone gene and the plasmid backbone (including ampicillin resistance gene and origin of replication) from the pC1 vector.

A library of DNA sequences was prepared by digesting the bacteriophage P1 cosmid P1-683 which contains the 5' half and upstream flanking sequence of the PSMA gene (40). Cosmid DNA was digested for various of times with the enzyme Tsp509I which cuts at AATT sites generating a range of partial digestion products. These were separated by agarose gel electrophoresis and fragments in the size range 1 to 2 kb recovered and cloned into the MfeI site of the pPSMentrap vector. A library of about 600 individual clones was picked.

Clones were grouped into 12 pools of 49 and DNA prepared from each pool using Qiagen columns and protocols. DNA (2.5 mg) from each pool was transfected into LNCaP cells in 3.5 cm dishes as previously described (1). After 48 to 72 hours, cell cultures were examined under a UV fluorescence microscope to identify any fluorescing cells. Positive pools were split into 7 by 7 matrices and DNA preparations made from the 7 clones in each row and each column. The transfections were repeated to identify positive sub-pools. Clones at the intersections of positive rows and columns were further screened individually to verify the expression of GFP. The three clones giving the strongest signals, #1, #3 and #4 were taken for further analysis.

Location and sequence analysis of enhancing fragments

The inserts from the clones were re-cloned into pBluescriptSK+ (pBKSEn3 and pBKSEn4) and the sequences of their ends determined. All clones were found to originate from the third intron of the PSMA gene as shown in Figure 2. The positions of both ends of clones #3 and #4 were identified as shown. The inserts in clones #3 and #4 were aligned in opposite orientations relative to the PSM promoter in the pPSMentrap vector as shown in Figure 3. The clones share a common overlapping sequence of 1044 bp and extend in total over 2,530 bp. The third clone, #1, derived from the same region, one end being 6 bp upstream of the end of clone #4 and it also contained the SpeI and HindIII sites contained in the region common to clones #3 and #4. It had, however, undergone some rearrangement on cloning and has not been further studied.

Function of PSMA enhancer region

The activity of the PSMA enhancer region was first identified by visual inspection of fluorescence intensity of cells transfected with clones carrying PSMA gene inserts upstream of the PSM promoter. In these preliminary experiments it was also noted that the enhancer (clone #4) did not appear to function in the bladder cell line BL13 (not shown). In order to provide for quantitative determination of promoter and enhancer function, enhancers #3 and #4 (hereafter designated En3 and En4) in combination with the PSM 1kb promoter were re-cloned into two different gene expression reporter systems.

Expression assayed in the pCAT3SAT system

The pCAT3SAT vector contains a modified bacterial chloramphenicol acetyl transferase reporter gene for determining promoter activity and a reference reporter gene, serine acetyl transferase, under the control of the RSV promoter in order to standardise CAT expression for transfection efficiency. It was prepared by cloning the serine acetyl transferase reporter gene from the pCATSAT plasmid (1) as a *Sall*/*Bam*HI fragment into *Bam*HI, *Sall* cut pCAT3 vector (Promega). Constructs, pPSM1k-C3S and pEn4PSM1k-C3S, containing the PSM promoter with or without the PSM enhancer fragment 4 (En4) were prepared by cloning the PSM enhancer/promoter fragments as *Sall*/*Pst*I fragments from the pPSMentrap vector into pCAT3SAT cut at the *Xho*I and *Pst*I sites in the polylinker upstream of the CAT gene (Figure 4). A control construct containing the RSV promoter, pRSV-C3S, was also prepared by blunt end ligation of a *Nae*I to *Sac*I fragment from pCATSAT (1) into the *Nhe*I site of pCAT3SAT (Figure 4). Cell lines were transfected with the different constructs and CAT and SAT activities determined after 48 h as described (1). The normalised expression data are shown in Figure 5.

In LNCaP cells an enhancement of expression of approximately 50 fold (from 0.33% to 15.7% of the activity of the RSV promoter) was seen when the En4 fragment was present upstream of the 1 kb PSM promoter. This expression showed a high level of specificity for LNCaP cells that express PSMA. Another prostate cell line, PC3, showed very low levels of expression from the PSM promoter either in the presence or absence of the enhancer. No expression above background was seen for three non-prostate cell lines (MCF-7, a breast cancer line, human embryonic kidney cells (HEK293) and the liver line HepG2). Low and variable expression was seen in a second breast cancer cell line T47-D2, with the enhancer/promoter construct showing about 10% of the activity seen in LNCaP cells.

Expression assayed in the luciferase pGL3 system

Because of the low activity of the PSM 1kb promoter in the CAT assay system, promoter and enhancer sequences were cloned into the pGL3 vector (Promega) which contains the luciferase reporter gene. The structure of the clones is shown in Figure 6. pPSM1k-GL3 and pEn4PSM1k-GL3 were prepared by cloning *Kpn*I to *Xba*I fragments from pPSM1k-C3S and pEn4PSM1k-C3S respectively into pGL3 cut with *Kpn*I and *Nhe*I. pEn3PSM1k-GL3 was prepared

by cloning the KpnI to NheI enhancer fragment of pEn3PSMentrap into pEn4PSM1k-GL3 cut with KpnI and NheI. To assay activity, mixtures of each pGL3 construct and the reference plasmid pRSVCAT (1) were transfected into a variety of cell lines by standard procedures as described previously (1). DNA concentrations were determined by image analysis of ethidium bromide stained gels and master mixes prepared in the ratio of 1.5 μ g of pGL3 construct to 1 μ g of pRSVCAT. The same master mixes were used for transfections into all cell lines. Cells were transfected with 2.5 μ g of DNA mixes using standard procedures (1) and expression assayed after 48 hr. Extracts were prepared and luciferase activity determined using the Luciferase Assay System (Promega). CAT activities were determined as previously described. Luciferase expression levels were standardised with respect to the pRSVCAT reference plasmid and then standardised activities expressed as a proportion of that of pRSV-GL3/pRSVCAT (Figure 7).

In LNCaP cells expression from the PSM 1k promoter was strongly enhanced by both En3 and En4 enhancer sequences (about 260 fold) with expression levels directed by pEn3PSM1k and pEn4PSM1k being 15 and 15.7% that of the RSV promoter. In the non-PSMA-expressing prostate cell line PC3 a low level of enhancement (3.7 and 5.2 fold for En3 and En4 respectively) was seen, while there was no enhancer function in the other non-expressing prostate line, DU145. For a range of non-prostate cell lines tested, HepG2 liver cells, MRC5 primary lung fibroblasts, BL13 bladder carcinoma and human embryonic kidney HEK293 cells, essentially no activity was seen for the PSMA enhancer/promoter or promoter alone constructs. Activity is thus highly specific for the expressing prostate cell line LNCaP with partial enhancer function in one non-expressing prostate cell line PC-3.

Characterisation of the enhancer element

To determine the extent of sequences required to provide enhancer activity a construct was prepared that contained all the sequences encompassed by clones En3 and En4 as well as constructs containing the overlapping region present in both clones (see Figure 6). pEn3+4PSM1k-GL3 was prepared by cloning a KpnI to NdeI restriction fragment from pBKSEn3 into pEn4PSM1k-GL3 cut with KpnI and NdeI. Clone pOverlappen3/4a was prepared by cloning the SalI to HindIII fragment from pEn3PSMentrap into pBluescriptSK+, subsequently cloning the HindIII fragment from

pEn4PSMentrap into the *Hind*III site of the intermediate vector and verifying that it was in the correct orientation. The overlapping enhancer fragment was then cloned as a *Kpn*I to *Eco*RI fragment in front of the PSM 1kb promoter in pPSM1k-GL3 cut with *Kpn*I and *Eco*RI. A construct with the overlapping region in the opposite orientation relative to the PSM promoter was likewise prepared by first cloning the *Sal*I to *Hind*III fragment from pEn4PSMentrap into pBluescriptSK+ followed by the *Hind*III fragment from pEn3PSMentrap and then cloning the overlap region in front of the PSM promoter as a *Kpn*I to *Eco*RI fragment.

The effectiveness of these constructs was compared with that of the PSM1k promoter alone and the En4/PSM1k promoter by transfection (as above) into LNCaP cells. Clones containing either orientation of the overlap region gave rise to expression levels similar to those containing En 4 sequences. The construct containing the whole region encompassed by enhancers 3 and 4, however, gave significantly stronger expression. The level of expression was about half that of the RSV promoter.

PSMA enhancer action on other promoters

The properties of the enhancer were further assessed by linking it to other promoters, both those active primarily in prostate cells, PSA and probasin, and a non-tissue-specific promoter, that of the herpesvirus thymidine kinase gene (TK). The structures of these promoter regions are shown in Figure 8. For the PSA and probasin constructs the enhancer region, En4, was cloned as an *Nhe*I fragment from the pEn4PSM1k-C3S plasmid into the *Xba*I-cut plasmids pPSA630 CATSAT and pPb430 CATSAT respectively (by partial digestion with *Xba*I for the probasin construct). pPSA630CATSAT and pPb430 CATSAT have been described previously (1). The plasmid pTKCATSAT.1 was prepared by cloning the TK promoter region, bases -101 to +59, as a *Sal*I to *Xho*I fragment into the *Sal*I-cut vector pCATSAT.1 (1)[pCATSAT.1 is a derivative of pCATSAT (1) in which *Sal*I, *Pst*II and *Xho*I sites present upstream of the RSV promoter were removed or destroyed by *Xho*I and partial *Sal*I digestion and religation]. pEn4TKCATSAT was prepared by cloning the *Sal*I to *Bgl*II enhancer-containing fragment from pEn4PSMentrap into pTKCATSAT.1 cut with *Sal*I and partially cut with *Bam*HI.

All six plasmids were transfected into a number of cell lines and CAT and SAT reporter gene expression determined as described (1). Expression

levels were standardised against that of the RSV promoter determined by transfection of a standard mixture of pRSVCAT and pRSVSAT plasmids as described (1). Results are shown in Figure 9a & b.

In LNCaP cells strong enhancement of the PSA, probasin and TK promoters was seen, with that for probasin being strongest. Levels of expression for all enhancer constructs were 2 to 3 times that of the RSV promoter. Since all promoters achieved similar levels of expression in the presence of the enhancer the "fold-enhancement" shown probably reflects differences in the level of non-enhanced expression from the different promoters.

In PC3 prostate cells, which do not express PSMA, much reduced enhancement was seen, being 5 to 16 fold for the different promoters. This is similar to the results seen when the enhancer was joined with its own PSM promoter. Thus it appears that PC3 cells contain some factors that can interact with the PSM enhancer to activate transcription, but lack others, or do not have sufficient levels, to enable full enhancer function as is seen in LNCaP cells.

For the non-prostate cell lines, no enhancement was seen in HepG2 liver or BL13 bladder cells. Enhancement was seen in the embryonic kidney HEK293 cells. Low level enhancement (1.4, 1.5 fold) was seen for the PSA and TK promoters, while there was a stronger 9 fold enhancement of the probasin promoter. No enhancement by En4 of its homologous PSM promoter was seen in HEK293 cells (Figure 7). Since the proximal kidney tubules are a site of low level PSMA expression, the expression seen in HEK293 cells may be biologically meaningful.

PSM Enhancer Function Does not Require Androgens

The androgen requirement for activity of the PSM enhancer (En4) was studied when it was linked to two highly androgen-inducible promoters, those of the probasin and PSA genes and one constitutive promoter, TK. LNCaP cells were transfected with plasmid constructs using media that had been charcoal stripped to remove androgens. Cells were maintained in androgen-free medium or incubated in the presence of the non-metabolizable androgen analogue, R1881 added to 0.28 nM (1). For all promoters strong enhancement of expression was seen whether or not androgen was present in the medium. However, for all three constructs containing the PSM enhancer the level of expression actually decreased upon androgen addition. This suggests that the

enhancer may contain sequences mediating the observed androgen-suppression of the endogenous PSMA gene.

Sequences required for enhancer function

In order to determine what sequence regions were critical for enhancer function a series of constructs were prepared in which different fragments from the PSME region were placed in front of the PSM promoter in the pPSM1k-GL3 plasmid. The sequences included in each construct are shown in the table below. The orientation of the enhancer sequences relative to the promoter is indicated as either F (forward, as for pEn4PSM1k-GL3) or R (reverse, as for pEn3PSM1k-GL3). Activity of these constructs was assayed following transfection into LNCaP cells along with the pRSVCAT control plasmid. Extracts were prepared and assayed 48 hr after transfection, luciferase activity normalised using the activity of the co-transfected pRSVCAT plasmid and expressed relative to that of pRSV-GL3 (Table below).

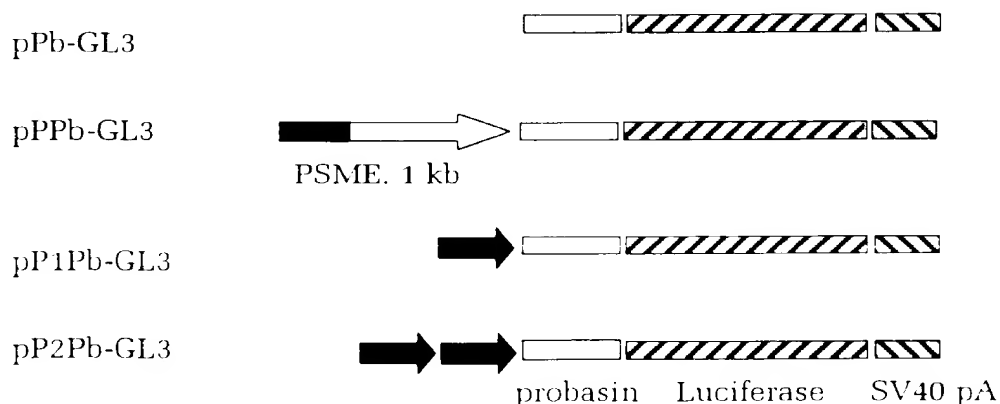
Construct	Enhancer sequences	Activity in LNCaP cells (% RSV)
pPSM1k-GL3		0.2
pEn4PSM1k-GL3	14760 - 16575 F	16.0
pEn3PSM1k-GL3	14045 - 15804 R	15.7
pEn3 + 4PSM1k-GL3	14045 - 16575 F	39
pEn3/4aPSM1k-GL3	14760 - 15804 F	25
pEn3/4bPSM1k-GL3	15804 - 14760 R	21
pEn4Sal/HindIII PSM1k-GL3	14760 - 15374 F	20
pEn3Sal/HindIII PSM1k-GL3	15804 - 15369 R	0.1
pEnO2/770SpeIPSM1k-GL3	14760 - 15530 F	24
pEnO2/2592NsiIPSM1k-GL3	14760 - 15352 F	22
pEnO2/445MscIPSM1k-GL3	14760 - 15205 F	18
pEnO2/331SmaIPSM1k-GL3	14760 - 15091 F	26
pEnO2/168NdeIPSM1k-GL3	14760 - 14930 F	6
pEnO1/722SmaIPSM1k-GL3	15092 - 15804 R	0.3
pEnO1/886NdeIPSM1k-GL3	14925 - 15804 R	0.4

These data indicate that most of the enhancer activity is contained within the 331 bp region encompassing bases 14760 to 15091. This region shows similar activity (26% that of RSV) to the En3 and En4 clones and to the approximately 1kb region shared between them. Deletion from the 1 kb overlap region of either the left half or the entire 331 bp region (constructs pEnO1/722SmaIPSM1k-GL3 and pEnO1/886NdeIIPSM1k-GL3) eliminates enhancer activity, showing that this region is essential for activity. Elimination of the right half of the 331 bp region, leaving just 170 bp covering bases 14760 to 14930, leads to a marked reduction in activity.

Thus bases 14760 to 14930 are essential for PSME function, but sequences extending from 14760 to 15091, provide for much stronger enhancer activity. The sequence of the region is shown in Figure 11.

Tandem enhancer sequences provide for greater activity

A series of constructs were prepared in which the probasin promoter, with or without PSM enhancer fragments was subcloned in front of the luciferase reporter gene in the pGL3 vector. The structure of the constructs is shown below. The 430 bp probasin promoter fragment has been described previously (1) and was re-cloned from the pPB-CS plasmid (see Figure 8). pPPb-GL3 contains the 1 kb overlapping enhancer region (bases 14760 to 15804). pP1Pb-GL3 and pP2PPb-GL3 contain one or two copies respectively of the 331 bp enhancer region (bases 14760 to 15091). All enhancer sequences are in the forward orientation.



The constructs were transfected, along with an RSV-CAT control plasmid, into LNCaP, HEK293 or MCF-7 cells and expression measured in cell extracts prepared after 48 h incubation. Transfections were done in androgen-depleted media and luciferase activity corrected using the co-transfected RSV-CAT internal control.

Relative Luciferase Activity

	LNCaP	HEK293	MCF-7
pPb-GL3	1.45	2.36	0.36
pPPb-GL-3	246	2.17	1.09
pP1Pb-GL-3	346	3.2	0.73
pP2Pb-GL-3	798	1.8	5.75
pRSV-GL-3	318	277	107

Greatest expression in LNCaP cells is seen with the double enhancer construct, being 2 to 3 times greater than those constructs with a single copy of the enhancer. Specificity of expression is largely maintained in these transfection studies, though the pP2Pb-GL3 construct shows an elevated level of expression in MCF-7 cells.

Enhancer function in human umbilical artery cells

PSMA has been shown to be expressed in the neovasculature of a range of tumour types, but not in normal vasculature. We have determined, using reverse transcriptase PCR, that PSMA is expressed in endothelial cells derived from the human umbilical artery (HUAECs) (data not shown). Function of PSM regulatory sequences was therefore examined in these cells. The activity of the PSME coupled to the PSM 1 kb promoter was evaluated using a replication-defective adenovirus, human adenovirus Type 5, into which the expression cassette from the pPSMentrap vector with the En4 insert had been inserted. The virus, Ad525, carries the GFP gene with bovine growth hormone 3' polyadenylation sequences under the transcriptional control of PSME En4 sequences coupled to the PSM 1 kb promoter. A control virus, Ad526, in which the GFP gene was under the control of the ubiquitously-active promoter of the EIF-1a promoter was also used.

HuAECs were dissociated from umbilical arteries and cultured as described by Underwood and Bean (41) except that tissue culture dishes were coated with bovine, rather than chicken, fibronectin and media contained 10% bovine fetal serum. HUACs and control human lung fibroblast MRC-5 cells were plated at 4×10^4 cells per chamber in fibronectin-coated microscope slide chambers. The following day they were infected with 5×10^6 optical particle units per chamber of either Ad525 or Ad526. Expression of the GFP gene was monitored by fluorescence microscopy 6 days after infection.

Expression from the control virus was strong in both cell types. For the En4PSMGFP virus, however, strong expression was seen in HuAECs but no expression could be detected in MRC-5 cells (Figure 12). The Ad525 virus was also shown to express strongly in LNCaP cells (not shown). The combination of PSME and PSM promoter is thus able to specifically drive gene expression in these arterial cells that express the endogenous PSM gene.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 25th day of January 2000

COMMONWEALTH SCIENTIFIC AND
INDUSTRIAL RESEARCH
ORGANISATION

Patent Attorneys for the Applicant:

F B RICE & CO

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Figure 1

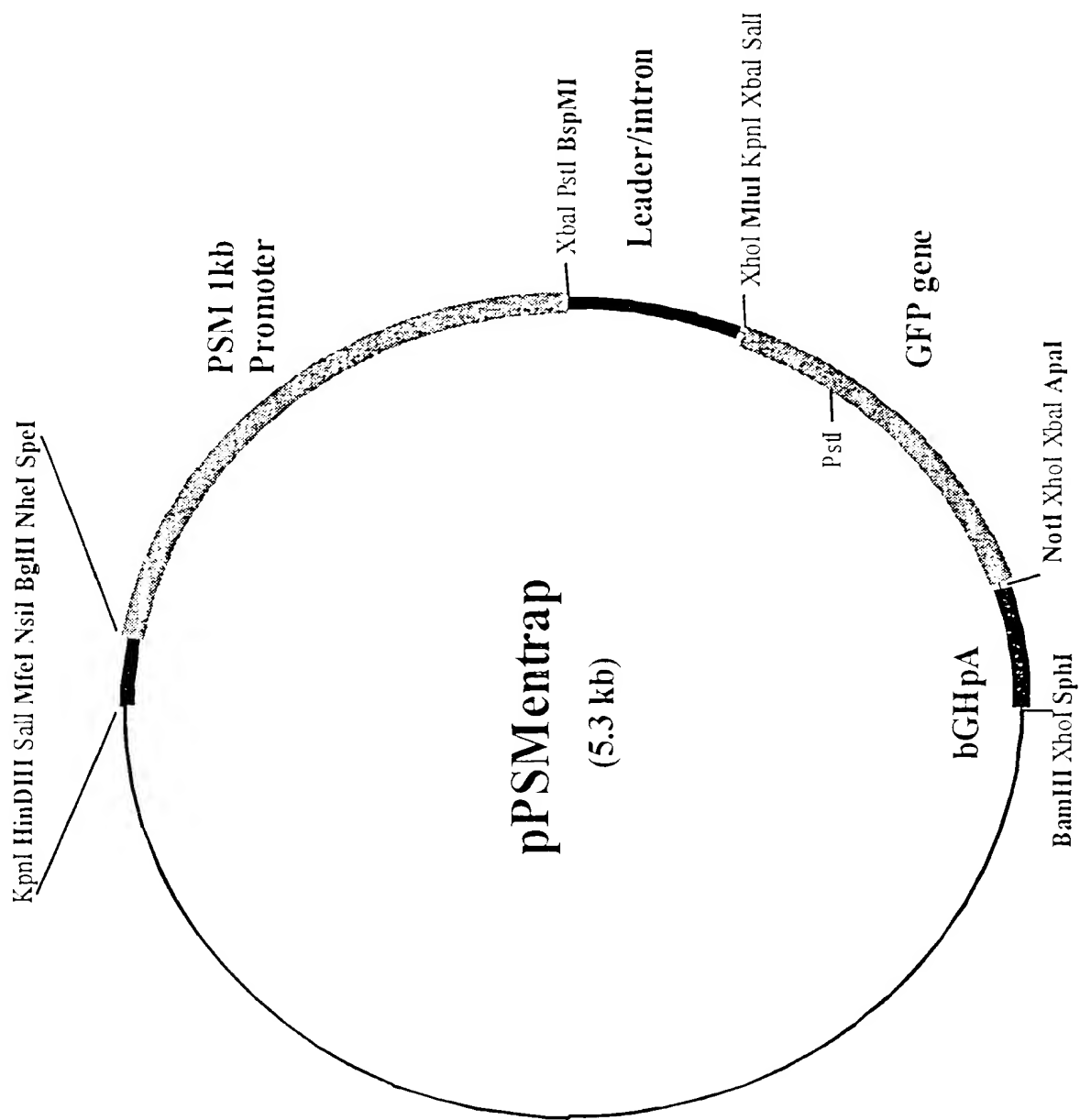


Figure 2 Location of cloned PSM enhancer fragments

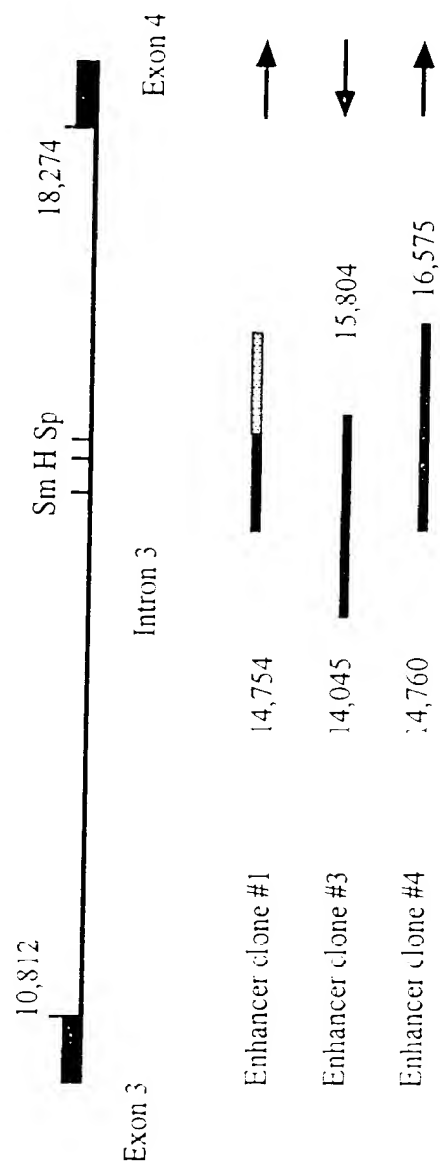


Figure 3 Promoter and Enhancer Inserts in pPSMentrap

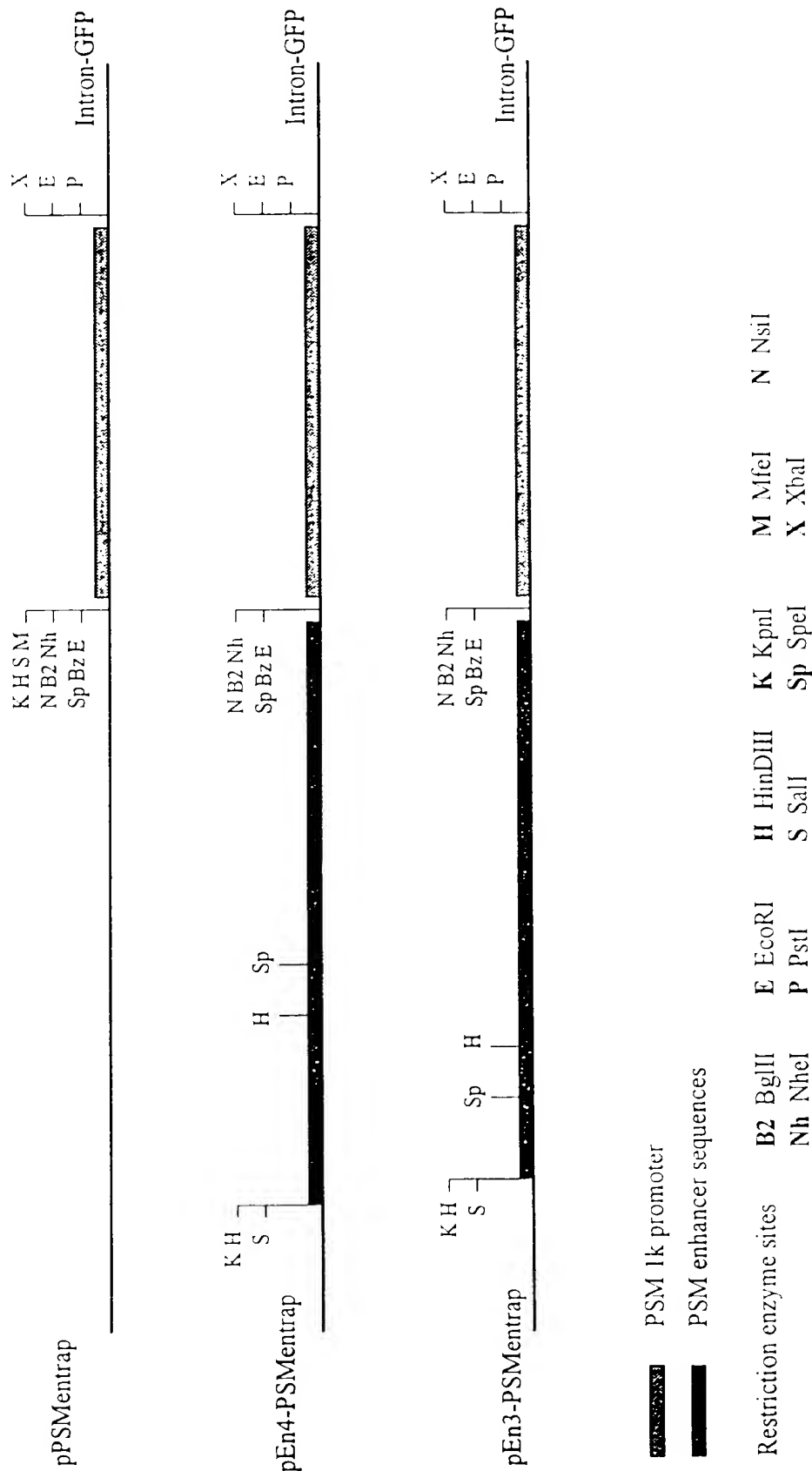
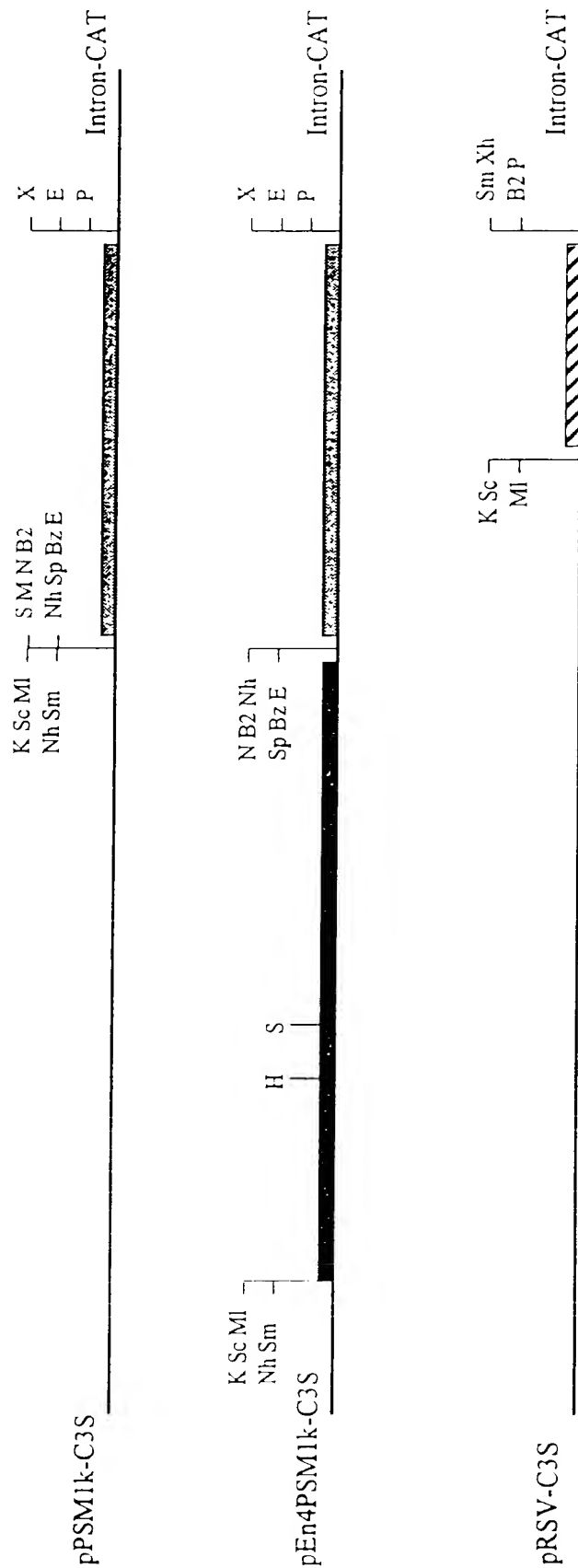


Figure 4 Promoter and Enhancer Inserts in pCAT3SAT



PSM 1k promoter

PSM enhancer sequences

RSV promoter

Restriction enzyme sites.

B2 BglII	Bz BstZI	E EcoRI	H HinDIII	K KpnI	M MfeI	MI MluI	N NsiI
Nh NheI	P PstI	S SalI	Sc SacI	Sm SmaI	Sp SpeI	X XbaI	Xh XhoI

Figure 5 Relative CAT expression directed by the PSM Enhancer4/PSM1k promoter

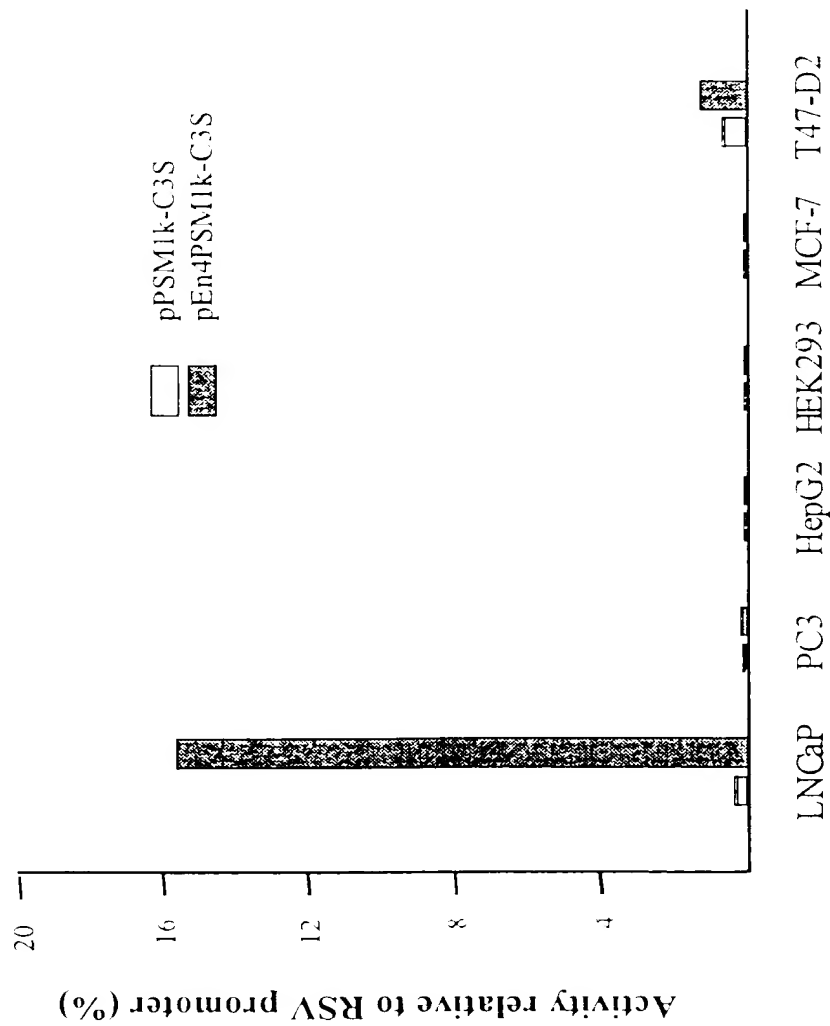


Figure 6 Promoter and Enhancer Inserts in pGL3

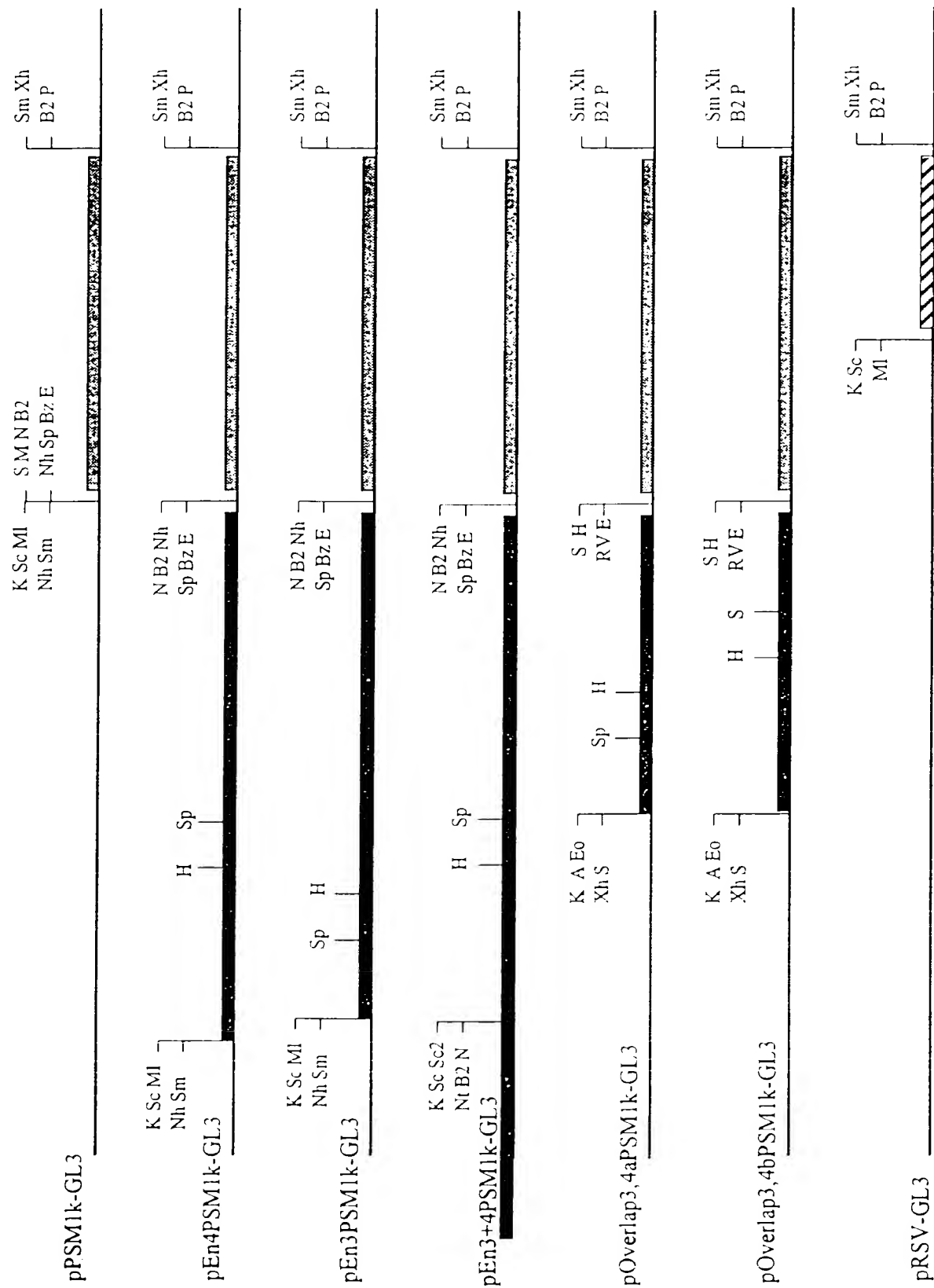


Figure 7 Relative luciferase expression of PSM enhancer/promoter constructs in the pGL-3 vector

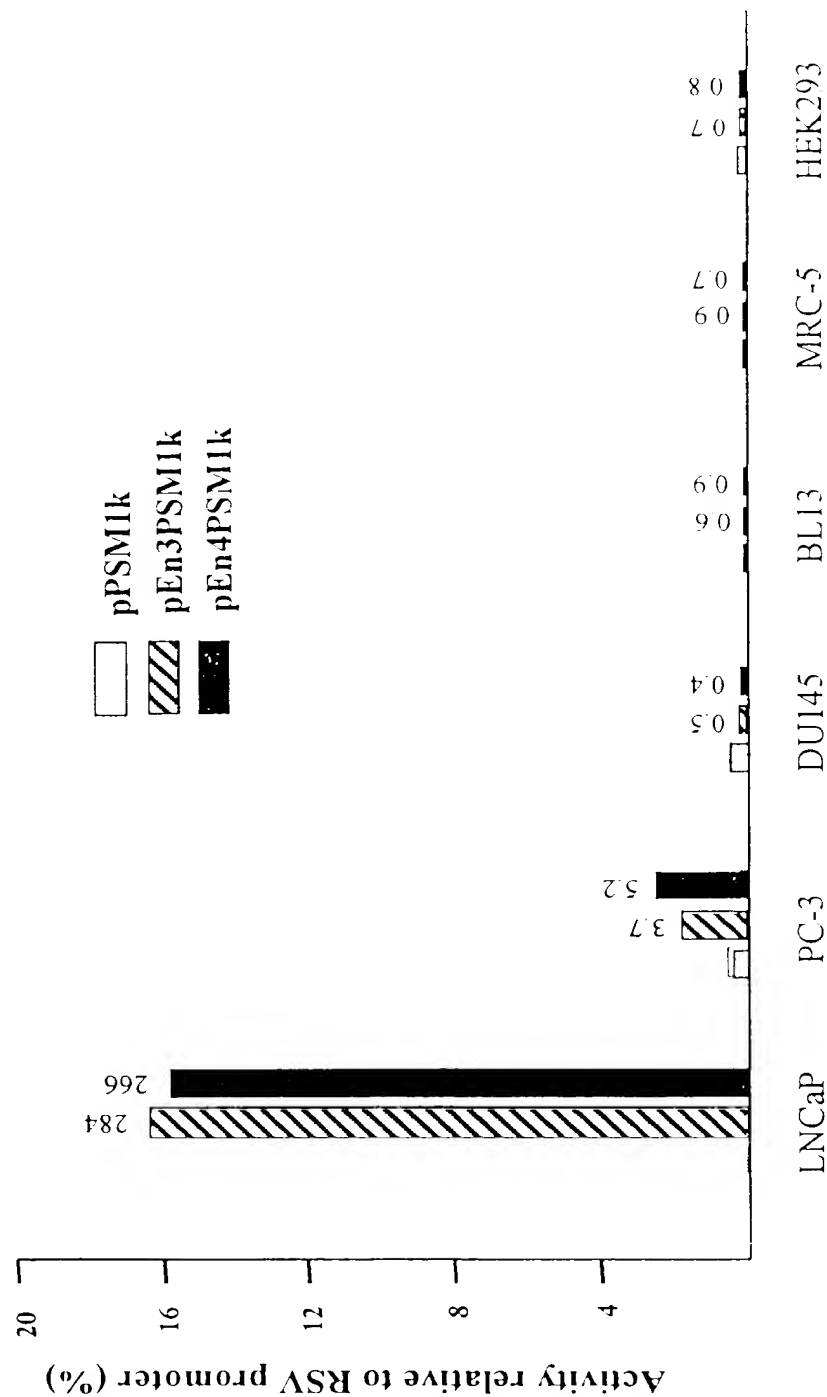
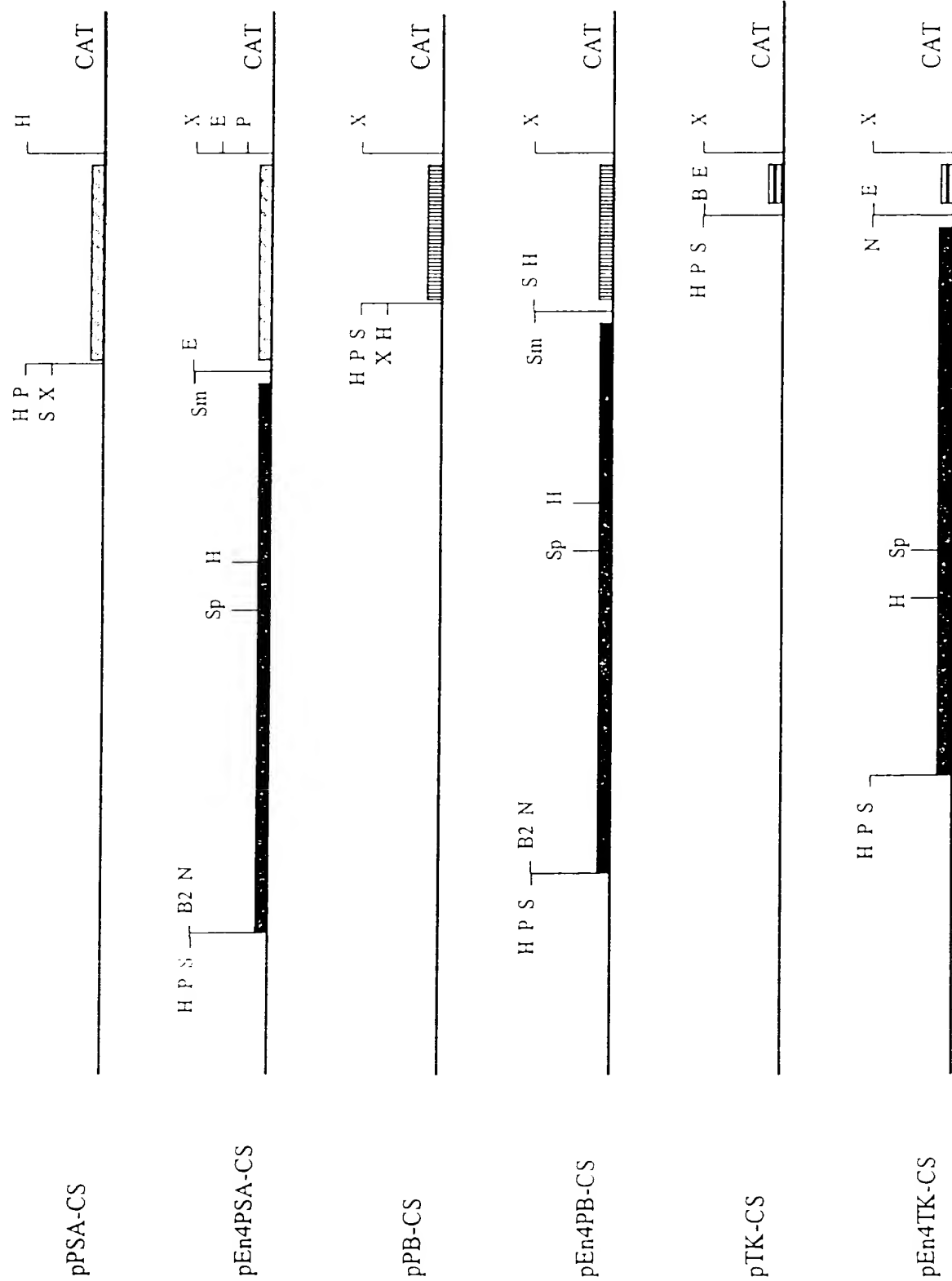


Figure 8 PSM Enhancer Constructs with other Promoters



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Assessing Probability of Ancestry Using Simple Sequence Repeat Profiles: Applications to Maize Hybrids and Inbreds

Donald A. Berry, Jon D. Seltzer, Chongqing Xie, Deanne L. Wright, and J. Stephen C. Smith. Determination of parentage is fundamental to the study of biology and to applications such as the identification of pedigrees. Limitations to studies of parentage have stemmed from the use of an insufficient number of hypervariable loci and mismatches of alleles that can be caused by mutation or by laboratory error and that can generate false exclusions. Furthermore, most studies of parentage have been limited to comparisons of small numbers of specific parent-progeny triplets thereby precluding large-scale surveys of candidates where there may be no prior knowledge of parentage. We present an algorithm that can determine probability of parentage in situations where there is no prior knowledge of pedigree and that is robust in the face of missing data or mistyped data. We present data from 54 maize hybrids and 54 maize inbreds. Accepted for publication March 11, 2002

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Manuscript received July 24, 2001

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DETERMINATION of parentage is fundamental to the study of reproductive and behavioral biology.

The increasing availability of highly discriminative genetic markers for many diverse species provides the potential to uniquely characterize individuals at numerous loci and to unambiguously resolve parentage where genealogical relationships are unknown, in error, or in dispute.

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Determination of parentage is fundamental to the study of biology and to applications such as the identification of pedigrees. Limitations to studies of parentage have stemmed from the use of an insufficient number of hypervariable loci and mismatches of alleles that can be caused by mutation or by laboratory error and that can generate false exclusions. Furthermore, most studies of parentage have been limited to comparisons of small numbers of specific parent-progeny triplets thereby precluding large-scale surveys of candidates where there may be no prior knowledge of parentage. We present an algorithm that can determine probability of parentage in situations where there is no prior knowledge of pedigree and that is robust in the face of missing data or mistyped data. We present data from 54 maize hybrids and 580 maize inbreds that were profiled using 193 SSR loci including simulations of additional levels of missing and mistyped data to demonstrate the utility and flexibility of this algorithm.

Most studies of pedigree have utilized exclusion analysis where the molecular marker genotypes of either one or a restricted number of potential triplets of offspring and putative parents are compared. Often the identity of the mother is not in question; the maternal profile is subtracted from that of the offspring and the deduced

(GOTZ and THALLER 1998; PRINIER *et al.* 2000; WHITE *et al.* 2000).

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and the like. Effective amounts of such diluent are obtained by using these amounts which are effective in obtaining

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This invention will be better understood from the experimental details which follow. However, one

Figure 10 Effect of androgens on enhancement of heterologous promoters by PSM En4

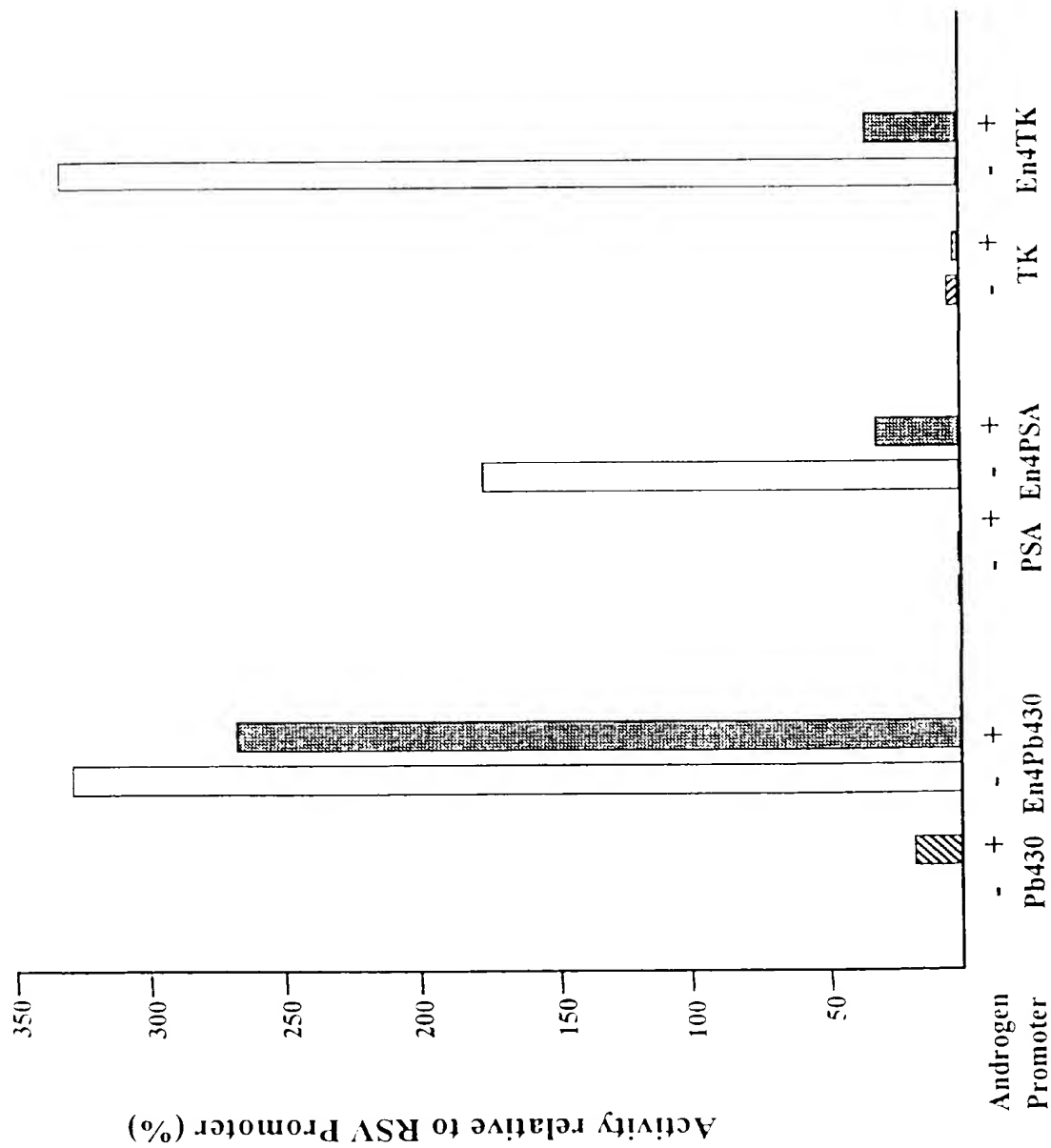


Figure 11 Sequence of 331 base pair core region of the PSME

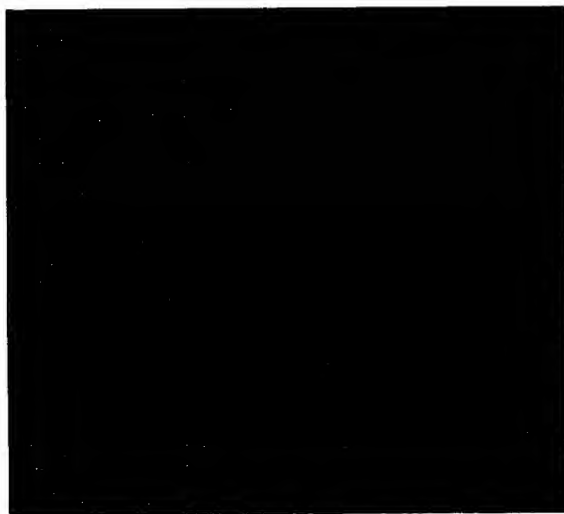
14760
AATTATTTTTTCCTTTAACCTTTCAAACCTCAAGGAAAACCAAGTTGGCCTTGACTCTGTTT
14820
GTGAAAAATTTTAAACTACTGGTTTAATTTCTTTATTGGTTGTAATATGACTATTTTACG
14880
TCATATAACAATTTTTATTGTTTGTTAAATGACTTTATTGTTTGTCATATGATAATTTTA
14940
TGTATAGAACAATTTTTATTGCTTGATATATGACTTTATTGTTATATGGCTATACAACT
15000
AGATTTTTTTGTTGTTTTTGACCGAGTCTTACTCTGTCACCCAGGCTGGAGTGTAATGGC
15060
ATGGTCTCAGCTCACTGCAACCTCCGCCTCCCGGG

The NdeI site 168 base pairs from the start of the core enhancer is underlined

Figure 12 GFP expression directed by the PSM enhancer/promoter



HUAECs infected with Ad525



MRC-5 cells infected with Ad525